

Polymorph Characterization using Raman Spectroscopy in High Throughput Crystallization Studies

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Introduction

Solid dose drugs make up a considerable part of the pharmaceutical market. A solid dose form of a drug can offer long shelf life, convenient ways of drug delivery, and can be packaged into a variety of forms such as capsules, tablets, gel caps, and patches.

Polymorphism is the ability of a molecule to exist in more than one stable or meta-stable crystalline state. The distinct crystalline forms commonly exhibit differences in pharmacologically relevant properties in solid dose form drugs. These properties include bioavailability, solubility, rate of absorption, duration, stability, manufacturability, and processibility. The factors influencing polymorphism are numerous, and include the solvents that are used in the formulation, the rate of recrystallization, salt selection, and temperature. Since polymorphism can have profound effects on the properties, and ultimately the manufacture of a compound under development, it is of critical importance to fully characterize and understand a compound's polymorphic behavior.

In response to this challenge, pharmaceutical companies are now extending their high throughput screening activities beyond the initial drug discovery stage into crystallization studies in a technique referred to as *high throughput crystallization* (HTC). Like high throughput screening, HTC experiments use small quantities of analytes, which are typically run in 96- and 384-well microtiter plates. New methods of analysis are now being developed to study the vast number of compounds generated by crystallization. While X-ray powder diffraction and thermal analysis have been successfully employed in HTC, new complementary techniques are strongly desired.

Any analytical method supporting HTC must meet most, if not all, of the following criteria: (1) the technique must be highly sensitive to differences in molecular geometric structure, (2) sampling should not require any special preparation, nor destroy the sample's geometric structure, (3) the measurement should be fast, and (4) the technique should be sensitive enough to be able to detect very small quantities of material.

Raman spectroscopy is an ideal technique for this application. With the advent of modern, highly automated spectrometers, Raman is rapidly becoming an indispensable tool for high throughput crystallization. Raman spectroscopy offers several particularly important advantages over other analytical techniques: (1) as a vibrational spectroscopy technique, Raman is very sensitive to molecular geometry, (2) Raman analysis requires little or no sample preparation, (3) collecting Raman spectra is much more rapid than

previous techniques, and (4) a Raman spectrum can easily be acquired from very small samples. In addition, Raman microscopy is extremely amenable to automation. This application note shows exciting new developments using a commercially available Raman microscope that are specifically designed to meet the needs of the high throughput crystallization community.

Experimental

All spectra in this report were acquired on the Thermo Scientific Nicolet™ *Almea*™ XR dispersive Raman spectrometer. The specific configuration of this Nicolet *Almea* XR uses depolarized 633 nm and 780 nm laser excitation sources, a Raman microscope equipped with high quality 10x-, 20x-, and 50x-long working distance objectives, a trinocular head with an attached color video camera for viewing, an automated x-y-z stage, and OMNIC™ software with optional Array Automation software. This instrument is designed to handle up to 1536 well microtiter plates conveniently, and is shown in Figure 1 and Figure 2. The Nicolet *Almea* XR is optionally available with the ValPro™ System Qualification package. This package provides a thorough set of qualification tools for instrument compliance with commonly accepted regulatory requirements. The ValPro System Qualification package includes all the necessary components for compliance with Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ) and Performance Qualification (PQ) testing.



Figure 1: Nicolet *Almea* XR Dispersive Raman Spectrometer with long working distance objectives, high resolution automated microscope stage, and well plate adapter

Key Words

- High Throughput Crystallization
- HTC
- Microtiter Well-plates
- Polymorphs
- Raman
- Salt Selection

Results and Discussion

The first step in the analysis of an HTC set of experiments is to identify the crystals of interest in a process called visual prescreening. HTC experiments rely upon using small quantities of materials, and are typically performed in 96-, 384- or even 1536-well microtiter plates. Since very small quantities of materials are used, the crystals will be distributed non-uniformly at the bottom of the wells of the microtiter plates. An example of a 96-well microtiter plate HTC experiment is shown in Figure 2.

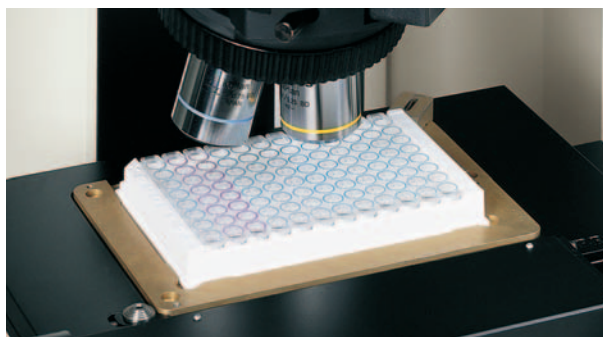


Figure 2: Microtiter plate on Nicolet Almega XR microscope stage. Recrystallization experiments were performed in each well to investigate the effects of different solvents and counter ions upon crystal formation. The plate was set up so that each well within a row had the same solvent but varied in counter ion present, while each well within a column used the same counter ion but varied in solvent used.

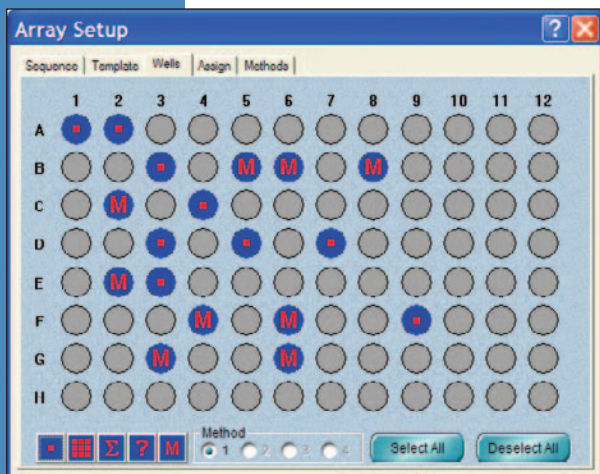
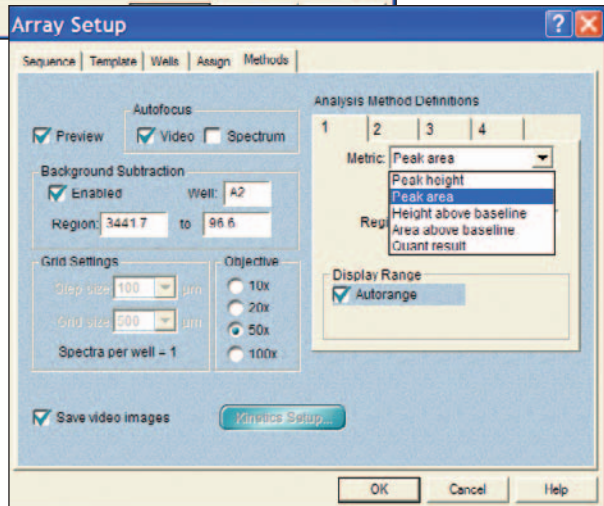


Figure 3: OMNIC Array Automation is designed to acquire spectra from samples positioned in an x-y array. Wells to be analyzed are selected from the screen on the left. Five different acquisition techniques are available and one of four different metrics can be used to analyze the spectrum from a selected well (below).



OMNIC Array Automation software provides extremely rapid screening with several different sampling options: a single measurement in the center of a microtiter well, a matrix of multiple measurements within a well, or points selected manually from the video image of the well. The latter option is particularly useful when the yield of crystals is low. In this case, the crystal or crystals are selected from the video image and coordinates for each crystal of interest are stored for spectral acquisition. The Array Setup screen shown in Figure 3 is used to customize the analysis by providing a number of automated options including autofocus, background auto-subtraction, and the choice of collecting a video image with the Raman acquisition.

Once crystals of interest have been identified, the Raman experimental conditions are defined (Figure 4), including exposure time, number of exposures, laser wavelength, and laser power.

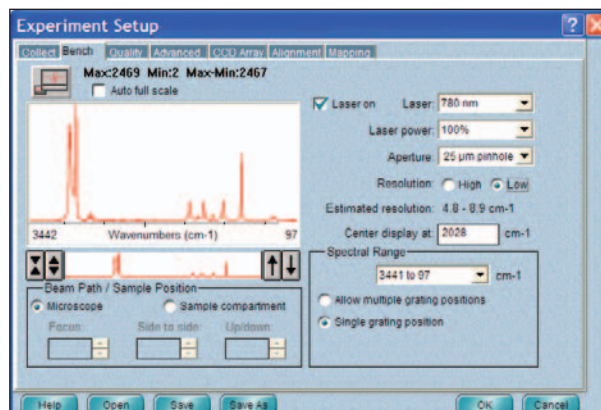
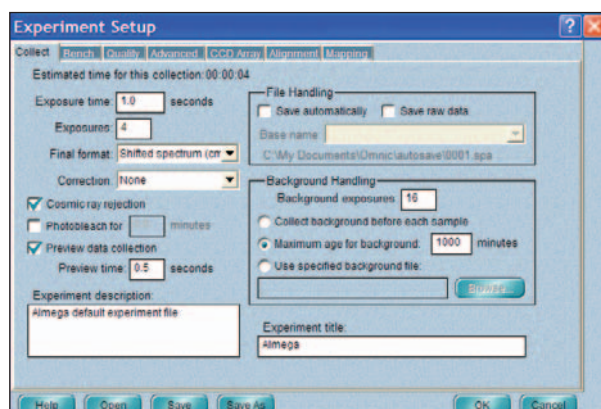


Figure 4: Raman Experimental Setup screen for setting collection and spectrometer parameters

Raman Measurement

It is well established that vibrational spectroscopy is an effective technique for characterizing polymorphs and solvates.¹ Raman spectroscopy, in particular, yields important information that can ultimately be related to the geometric structure of a molecule and its environment. Changes in crystal geometric structure or salt electrostatic environment will cause band shifts in the spectra. Gross changes in symmetry with different crystal packing geometries will cause overall band splitting, coalescence, or relative intensity changes.

Two precautions must be taken when using Raman as a technique for characterizing polymorphism. Many crystals exhibit an orientation-dependent interaction with plane polarized light. In these cases, apparent relative intensity differences between spectra of two identical polymorphs may simply be due to the fact that they are oriented differently with respect to the plane-polarized laser source. To illustrate this first problem, Raman measurements were performed on crystals of hydroquinone using vertically polarized laser excitation. These needle-like crystals were placed at four different orientations (Figure 5). Significant variation in the intensity of these bands is evident with orientation. This orientation-dependent artifact can effectively be eliminated by using a depolarized laser source. Figure 6 shows Raman spectra from the same hydroquinone crystals using a depolarized laser. In this figure the orientation-dependent changes are insignificant. The Nicolet Almega XR used in this study employed 633 nm and 780 nm depolarized lasers.

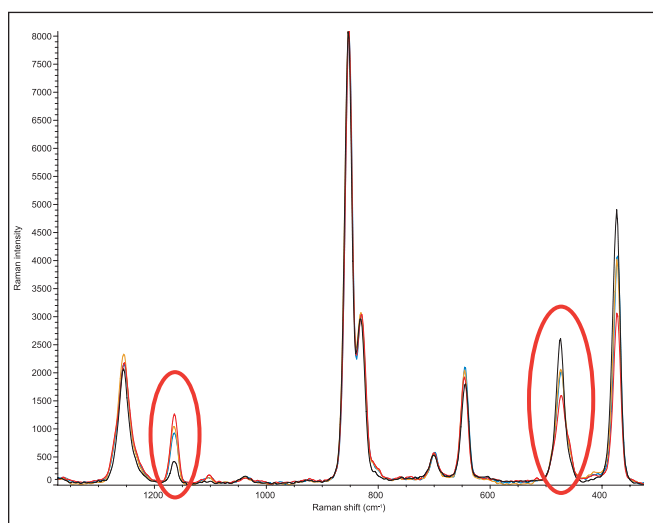


Figure 5: Raman spectra of hydroquinone using vertically polarized excitation observed at four different crystal orientations. The spectra are normalized to the 852 cm⁻¹ peak. Note the striking variation in peak height at the 472 and 1164 cm⁻¹ peaks.

The other problem that may be encountered is that of fluorescence, which may effectively overwhelm the Raman signal. On Nicolet Almega XR Raman systems, two depolarized lasers (633 nm and 780 nm) can be automatically exchanged within seconds to provide an alternate laser excitation wavelength selection, depending on whether fluorescence interference is present.

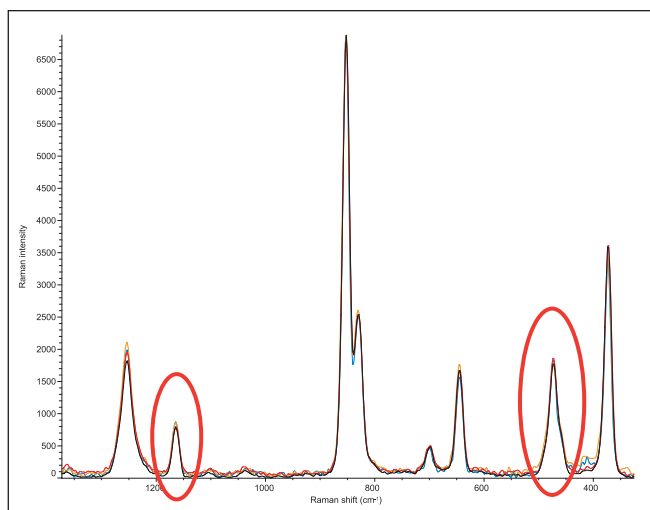


Figure 6: Raman spectra of the same hydroquinone crystals as in Figure 5, using depolarized excitation. These spectra are normalized to the 852 cm⁻¹ peak. Note the decrease in the variation of peak height at the 472 and 1164 cm⁻¹ peaks.

Figures 7 and 8 show the results of Raman measurements and brightfield microscopy obtained on two different crystalline forms of 5-Methyl-2-[(2-nitrophenyl)amino]-3-thiophenecarbonitrile.² Figure 7 demonstrates Raman's sensitivity in differentiating distinct crystalline forms. Spectra of isolated Forms A and B of this compound show distinct differences. This is particularly apparent in the region from 1200 cm⁻¹ – 1600 cm⁻¹ where band splitting and shifting are particularly noticeable in Form B.

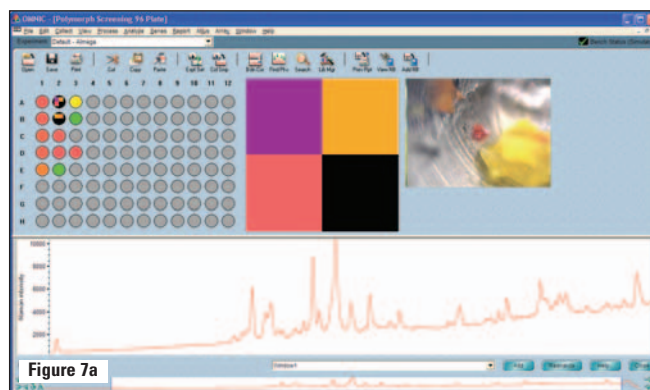


Figure 7a

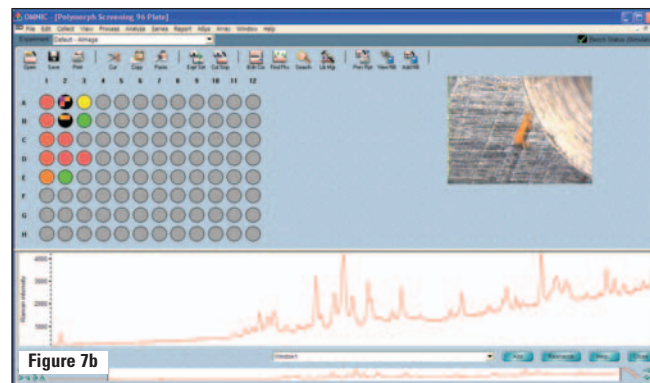


Figure 7b

Figure 7a and 7b: Results of single and multiple Raman measurement on a 96-well microtiter plate. Raman spectra and brightfield images were collected on the non-grey (prescreened) wells. (a) The red crystal (Form A) at the center of the brightfield image is associated with this Raman spectrum. (b) The Raman measurements on these orange needle-like crystals (Form B) in well B3 yield the spectrum presented in this window.

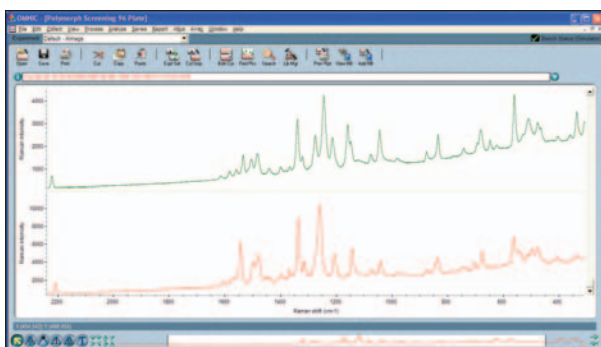


Figure 8. Raman spectrum of two polymorphs of 5-Methyl-2-[(2-nitrophenyl)amino]-3-thiophenecarbonitrile. The two crystalline Forms A and B exhibit distinctly different Raman spectra.

Within a collected data set there may exist some spectra that contain differing amounts of fluorescence background. In this case, it may be appropriate to apply some preprocessing to the spectra. OMNIC Array Automation software supports several preprocessing options, including the ability to generate derivative spectra and carry out the analysis on these spectra. It has been shown that the use of derivatives can greatly increase the ability to match or discriminate spectra. The Norris derivative, in particular, has been shown to be the most effective. It can be used to remove fluorescence background, better differentiate overlapped bands, and to distinguish small band shifts more clearly. Spectra processed using the Norris derivative are shown in Figure 9.

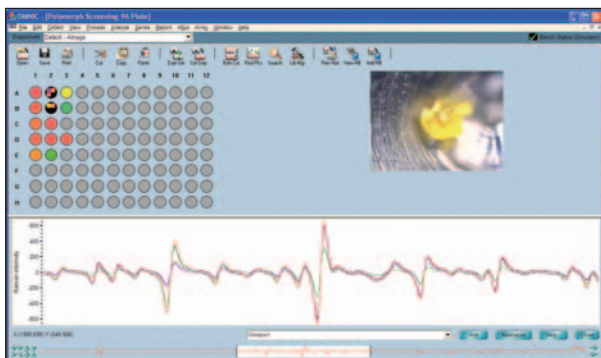


Figure 9: The results of applying the Norris derivative to the spectra presented in Figure 8. Since this function gives the rate of change of the intensity with respect to wavenumber, comparison of these spectra is particularly useful in assessing small band shifts. Spectra with no shift in peak positions have identical zero crossover points.

Interpretation of High Throughput Crystallization Data

The challenge of high throughput crystallization experiments is to compare large numbers of spectra to ensure that all variants in the screening have been identified. OMNIC Array Automation software dramatically reduces the analyst's work load by automating the tasks in the analysis. The primary objective is to identify potential new drug forms by performing experiments that investigate all possible conditions and possible outcomes. However, these experiments yield quantities of data that are overwhelming to analyze on a spectrum-by-spectrum basis. OMNIC Array Automation software provides powerful algorithms that dramatically reduce the analysis time. Using these software tools, the data acquired can rapidly be compared and grouped into classes based on spectral similarities that ultimately aid in the rapid discovery of new polymorphs.

Once the data set has been collected, it can be reanalyzed any number of times. A list of the reanalysis metrics is shown in Figure 10. In the example in this figure, the data have been reanalyzed using the *correlation metric*, which calculates the correlation between a selected reference spectrum and the remaining spectra in the data set. Color-coding provides a visual representation of the degree to which each well correlates with the reference. Wells coded in red in Figure 10 are more highly correlated with the reference well than are the wells coded in blue.

While the correlation metric is a useful tool, OMNIC Array Automation software contains another powerful means of data interpretation that does not require the use of a reference. The *group analysis metric* is based upon the cross correlation of all the data in the set with each other. It automatically performs cross correlation and assigns wells to groups according to the similarity of their spectra. This saves time by quickly drawing attention to wells containing potentially new forms. The time savings can be significant; for example, manual analysis of a 96-well microtiter plate may take an analyst four hours or more. Using the group analysis metric, the analysis time is reduced to as little as five minutes. Figure 12 shows an example of group analysis using a 384-well microtiter plate. Since group analysis provides information that is not based on prior knowledge of the data set, it is particularly useful for truly automated identification or screening of new compounds.

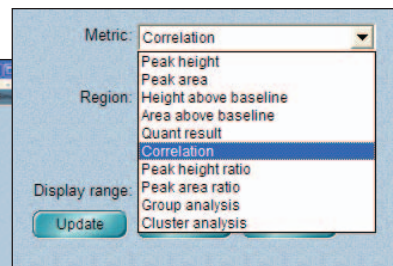
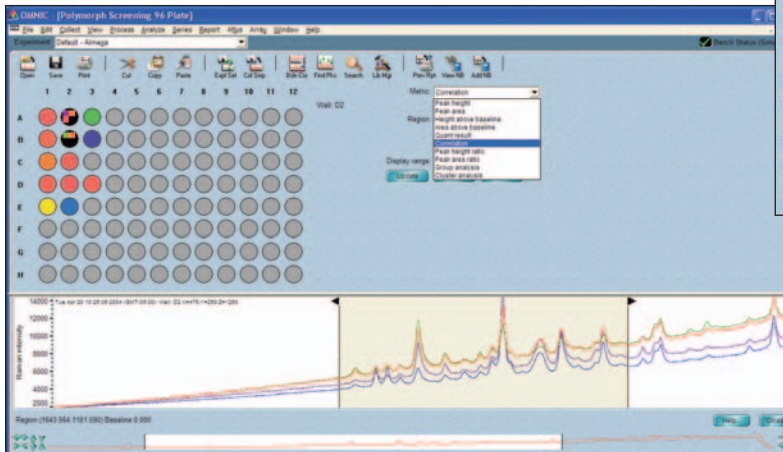


Figure 10: Correlation of a series of spectra from two polymorphs of 5-methyl-2-[(2-nitrophenyl)-amino]-3-thiophenecarbonitrile with a reference well (A1). OMNIC Array Automation color codes wells to indicate the degree to which the contents correlate with the reference well, making it easy to evaluate the results at a glance. Wells colored red (B1, C2, D1, D2, D3) indicates that they are highly correlated with the reference well. The correlation was measured over the region from 1650 cm⁻¹ to 1100 cm⁻¹.

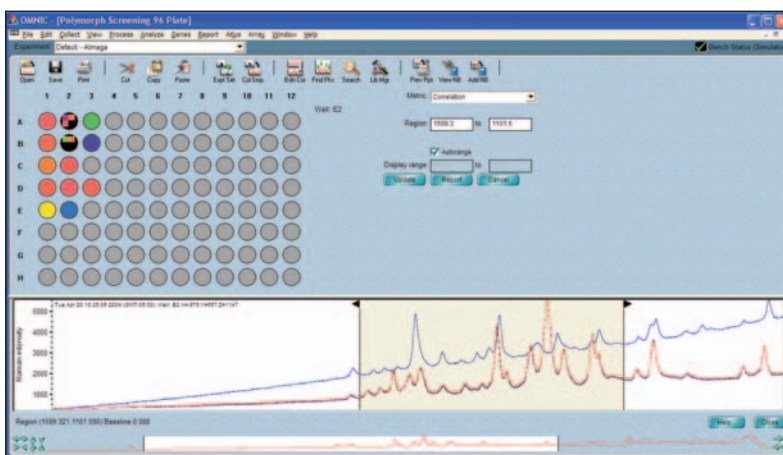


Figure 11. Same analysis as in Figure 10. Wells coded blue (E2) and purple (B3) have a much lower correlation coefficient. This is confirmed by an overlay of the three spectra (wells A1, E2, B3).

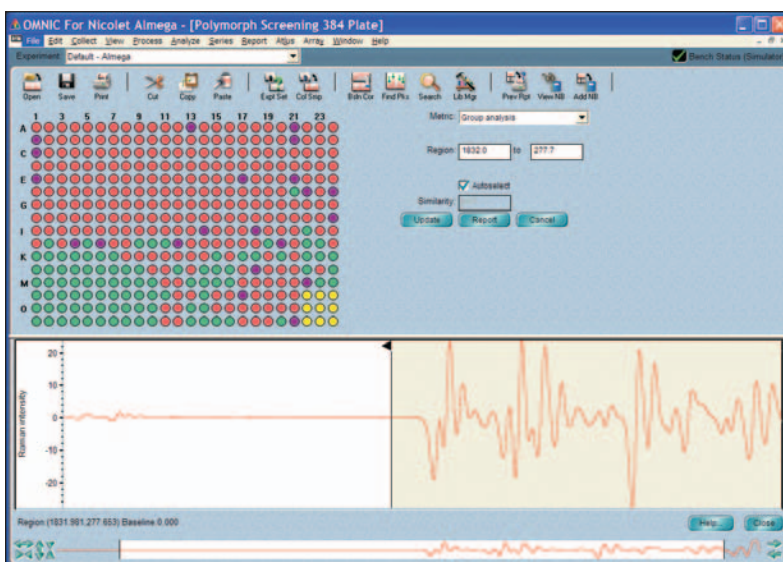


Figure 12: The results of performing a group analysis calculation after having applied the Norris derivative on the spectra from a 384-well microtiter plate in order to eliminate background interference. The analysis shows the presence of three groups (identified as red, green and yellow) and draws attention to the 21 ungrouped wells (purple).

Conclusion

This application note demonstrates that dispersive Raman spectroscopy using the Thermo Scientific Nicolet Almega XR, together with OMNIC Array Automation software, is a very sensitive probe for identifying polymorphic forms. As a technique, Raman is fast, requires very little sample preparation, can be used to characterize the smallest of samples, and is highly amenable to automation. Microtiter well-plate sampling capabilities and OMNIC Array Automation make the Nicolet Almega XR dispersive Raman spectrometer an efficient platform for high throughput crystallization studies.

References

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2. The authors wish to thank Professor Lian Yu, of the University of Wisconsin, School of Pharmacy, Division of Pharmaceutical Sciences, for providing for polymorphic compound 5-Methyl-2-[(2-nitrophenyl)amino]-3-thiophenecarbonitrile used in this work.

Suggested Configuration of the Nicolet Almega XR

- Nicolet Almega XR Dispersive Raman System
- 633 nm depolarized laser
- 780 nm depolarized laser
- High resolution, automated microscope stage
- Long working distance objectives
- Well-plate adapter
- OMNIC software with Array automation and TQ Analyst™ options
- Trinocular head with color video camera
- ValPro System Qualification package and OMNIC Digital Signature capability. Optional, recommended for use in regulated environments

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